

## Prognostic value of *K-ras* mutation status and subtypes in endoscopic ultrasound-guided fine-needle aspiration specimens from patients with unresectable pancreatic cancer

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### Abstract

**Background** Although recent reports indicate that *K-ras* mutation status is a biomarker that acts as a prognostic factor, only a few analyses of *K-ras* mutation subtypes have been published. In addition, there are no reports that analyze overall survival and prognostic factors according to *K-ras* mutation status and subtypes in only unresectable pancreatic cancer (PC) determined from tissues obtained by endoscopic ultrasound-guided fine-needle aspiration.

**Methods** We retrospectively analyzed 242 patients who were diagnosed as having unresectable PC with available histological diagnosis. Clinical data collected included sex, age, Eastern Cooperative Oncology Group performance

status, carbohydrate antigen (CA) 19-9, primary tumor location, stage (local or metastatic) according to TNM staging, first-line chemotherapy, *K-ras* mutation status and subtypes (G12D, G12V, and G12R), and overall survival. We analyzed the negative prognostic factors for reduced overall survival in unresectable PC patients using these data.

**Results** From multivariate analysis, CA19-9  $\geq 1000$  U/ml (hazard ratio [HR] 1.78, 95 % confidence interval [CI] 1.28–2.46,  $P < 0.01$ ), metastatic stage (HR 2.26, 95 % CI 1.58–3.24,  $P < 0.01$ ), and mutant-*K-ras* (HR 1.76, 95 % CI 1.03–3.01,  $P = 0.04$ ) were negative prognostic factors, indicating a reduced survival. Among the patients who had *K-ras* mutation subtypes, CA19-9  $\geq 1000$  U/ml (HR 1.65, 95 % CI 1.12–2.37,  $P < 0.01$ ), metastatic stage (HR 2.12, 95 % CI 1.44–3.14,  $P < 0.01$ ), and the presence of the G12D or G12R mutations (HR 1.60, 95 % CI 1.11–2.28) were negative prognostic factors for overall survival.

**Conclusions** *K-ras* mutation status and subtypes may be associated with survival duration in pancreatic cancer patients.

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### Introduction

The prognosis of pancreatic cancer (PC) is one of the poorest among all malignancies. The 5-year survival rate among all patients with PC is  $< 3.5$  % [1, 2]. Because PC has such a poor prognosis, it would be advantageous to accurately predict treatment outcomes to prevent over-treating patients who would have little benefit from chemotherapy. The prognostic factors for unresectable pancreatic adenocarcinoma include

serum carbohydrate antigen 19-9 (CA19-9), TNM staging, C-reactive protein, and baseline Karnofsky performance status (PS) [3–5]. Genetic abnormalities have been identified in PC patients, and *K-ras* mutations are found in approximately 90 % of PC patients [6, 7]. *K-ras* mutations have been examined as a possible prognostic factor for PC. Although recent reports indicate that *K-ras* mutation status is a biomarker that acts as a prognostic factor [8–10], only a few analyses of *K-ras* mutation subtypes have been published [11, 12]. These reports might include some false-negative cases, because the frequency of *K-ras* mutations was relatively low compared with previously reported frequencies [6, 7]. In addition, there have been no previously published analyses of the possible association between the prognosis of unresectable PC patients and the *K-ras* mutation subtype determined from tissues obtained by endoscopic ultrasound-guided fine-needle aspiration (EUS-FNA).

We previously detected *K-ras* mutations in 266 (87 %) of 307 PC patients from tissues obtained by EUS-FNA [13]. This percentage is similar to previously published rates of *K-ras* mutations [6, 7]; therefore, the data from our *K-ras* analysis appear to be reliable.

The aim of the present study was to use data from our previous study to retrospectively determine whether *K-ras* mutation subtypes are prognostic factors in PC.

## Methods

### Patient selection

The Institutional Review Board at Aichi Cancer Center Hospital approved this study of *K-ras* mutation status in 394 consecutive patients who underwent EUS-FNA of pancreatic masses between March 2004 and September 2009. We retrospectively included 242 patients with histologically diagnosed PC, who were deemed unresectable. Patients who underwent surgical resection were excluded. In addition, patients whose EUS-FNA results were negative for malignancy and *K-ras* mutation were excluded because of the possibility of false-negative results.

All patients provided written informed consent to all procedures associated with the study.

### Clinical data

The clinical data collected included sex, age, Eastern Cooperative Oncology Group (ECOG) performance status (PS), CA19-9, primary tumor location, stage (local or metastatic) according to TNM staging, first-line chemotherapy, *K-ras* mutation and subtypes, and overall survival (OS). The primary endpoint of this study was OS. Because

the G12D, G12V, and G12R subtypes comprised almost all of the *K-ras* mutations, we focused on these three subtypes in this study.

### EUS-FNA technique

We imaged the pancreas at 7.5-MHz frequency using a convex linear-array echoendoscope (GF-UGT240; Olympus Optical, Tokyo, Japan) connected to an ultrasound device (SSD5500; Aloka, Tokyo, Japan) and a 22-gauge needle (NA-10J or NA-11J-KB, Olympus Optical; or an EchoTip-Ultra Needle; Cook Medical, Limerick, Ireland), as previously described [14, 15]. The aspirated material was separated for cytopathological evaluation, cell-block preparation, and *K-ras* mutation analysis. The aspirated material was immediately evaluated (Diff Quick Staining, Baxter, Miami, FL, USA) by a cytopathologist and/or cytotechnologist for rapid diagnosis. Material was directly fixed in 10 % formalin in a standard specimen bottle, centrifuged, and then embedded in paraffin for cell-block analysis. Sections were visualized by hematoxylin and eosin (HE) staining, as well as immunohistochemical staining if necessary.

### Analysis of *K-ras* mutations

The genetic analysis was performed with material obtained from EUS-FNA (either fresh specimens or paraffin-embedded tissues). Total RNA was extracted from the fresh specimens, and mutational analysis was performed using reverse transcription polymerase chain reaction (RT-PCR)-coupled direct sequencing methods, as described previously [16]. When the direct sequencing displayed no mutational signal although the cytological diagnosis was atypical cells, suspicious of adenocarcinoma, we further investigated *K-ras* mutation, using the corresponding cell-block slides, by performing a Cycleave PCR assay (TAKARA, Co., Ltd., Ohtsu, Japan) [17]. This assay is highly sensitive and can detect as little as 5 % tumor cells mixed with normal tissues.

### Statistical analysis

Continuous variables were expressed as median values. Incidences and concordance between groups were compared using the  $\chi^2$  test, Mann–Whitney *U*-test, and Kruskal–Wallis test where appropriate. OS was measured from the day that PC was histologically diagnosed by EUS-FNA to the time of death or last follow-up examination. Survival rates were calculated using the Kaplan–Meier method [18]. The stratified log-rank test was used to compare survival curves, and censored data were taken into account [19]. Both univariate and multivariate analyses were used to

examine potential prognostic factors. The Cox proportional hazards model was used to determine the most significant variables contributing to survival [20]. Differences with a *P* value of less than 0.05 were considered statistically significant. All analyses were performed with SPSS version 11.0 (SPSS, Chicago, IL, USA) statistical software.

## Results

### Patient characteristics

The patient characteristics according to *K-ras* mutation status in 242 patients with unresectable PC are shown in Table 1. Among the 242 patients, 214 (88.4 %) patients had mutant *K-ras*, and 28 (11.6 %) patients had wild-type *K-ras*. Among the 214 patients with *K-ras* mutations, the most common subtypes were G12D (92 patients; 42.9 %), G12V (85 patients; 39.7 %), and G12R (17 patients; 7.9 %). Less common *K-ras* mutations were in codon 13 (*n* = 1) or codon 61 (*n* = 5), while 10 patients had unknown *K-ras* mutations. There were no significant differences between the sex distribution or median ages of the patients with different mutation subtypes, or between patients with mutant and wild-type *K-ras* genes. Sixteen patients had a PS of  $\geq 2$  (6.6 %) and 226 had a PS of  $< 2$  (93.4 %). CA19-9 levels, primary tumor location, and tumor stage (local or metastatic) were not related to the different mutation subtypes, or to the presence of mutant or wild-type-*K-ras* genes. A total of 86 % of patients with the wild-type-*K-ras* gene, and 80 % of patients with the mutant *K-ras* gene were treated with gemcitabine (GEM). Patients who were not treated with GEM received titanium silicate (TS)-1, a combination of 5-fluorouracil and radiation, or no therapy. There were no significant differences in the treatment modalities among the different mutation subtype groups, or between the mutant and wild-type-*K-ras* gene groups.

### Survival according to *K-ras* mutation and subtypes

Overall survival was examined with Kaplan–Meier plots and log-rank tests, as shown in Figs. 1 and 2. The median OS was 261 days for the entire group of PC patients. When analyzed by *K-ras* mutation status, the median OS was significantly longer in the wild-type group (479 days) than in the mutant group (255 days; *P* = 0.03). Moreover, the median OS values were significantly different (*P* < 0.01) among the patients who had the three most common *K-ras* mutations: G12D (242 days), G12 V (338 days), and G12R (204 days). Patients who had the G12 V mutation had a significantly longer OS than patients with G12D (*P* = 0.01) or G12R (*P* < 0.01). There was no statistically

significant difference between the OS of the G12D and G12R subgroups (*P* = 0.13).

### Univariate and multivariate analyses

The results of univariate and multivariate analyses are listed in Table 2 (according to wild-type vs mutant) and Table 3 (according to *K-ras* mutation subtype). Univariate analysis revealed that the negative prognostic factors for OS were PS  $\geq 2$  (hazard ratio [HR] 2.58, 95 % confidence interval [CI] 1.46–4.55, *P* < 0.01), CA19-9  $\geq 1000$  U/ml (HR 1.95, 95 % CI 1.42–2.69, *P* < 0.01), and metastatic stage (HR 2.44, 95 % CI 1.72–3.45, *P* < 0.01). Patients with a mutant *K-ras* gene had a shorter survival than patients with the wild-type gene (HR 1.78, 95 % CI 1.08–2.95, *P* = 0.03). Interestingly, patients with the G12D or G12R mutation had a shorter survival than patients with the G12V mutation (HR 1.72, 95 % CI 1.22–2.43, *P* < 0.01).

The multivariate analysis performed according to *K-ras* mutation revealed that CA19-9  $\geq 1000$  U/ml (HR 1.78, 95 % CI 1.28–2.46, *P* < 0.01) and metastatic stage (HR 2.26, 95 % CI 1.58–3.24, *P* < 0.01) were negative prognostic factors for OS. In addition, the presence of a mutant *K-ras* gene (HR 1.76, 95 % CI 1.03–3.01, *P* = 0.04) was a negative prognostic factor for OS. According to the *K-ras* mutation subtype, CA19-9  $\geq 1000$  U/ml (HR 1.65, 95 % CI 1.12–2.37, *P* < 0.01) and metastatic stage (HR 2.12, 95 % CI 1.44–3.14, *P* < 0.01) were also negative prognostic factors for OS. Regarding *K-ras* mutation subtypes, G12D and G12R (HR 1.60, 95 % CI 1.11–2.28) were negative prognostic factors for OS.

## Discussion

Various genetic abnormalities in *K-ras*, *p53*, *p16*, and *DPC4* have been demonstrated in PC [6, 7, 21–23]. *K-ras* is the most common mutationally activated human oncoprotein, and *K-ras* mutations account for 90 % of the mutations in PC. In advanced colon cancer, *K-ras* mutation status and subtypes are extremely useful in predicting the therapeutic efficacy of chemotherapy and identifying patients with poor prognosis [24]. Therefore, we used our previously collected data on the incidence and subtypes of *K-ras* mutation in PC [13] to clarify the relationship between *K-ras* mutations and clinicopathological features. There were no significant differences in the treatment modalities used for the patients with wild-type and those with mutant *K-ras*. In addition, in patients thought to have a high response rate to chemotherapy based on tumor markers and diagnostic imaging data, the wild-type gene was present in 29 % and the mutant type in 14 %. Divided

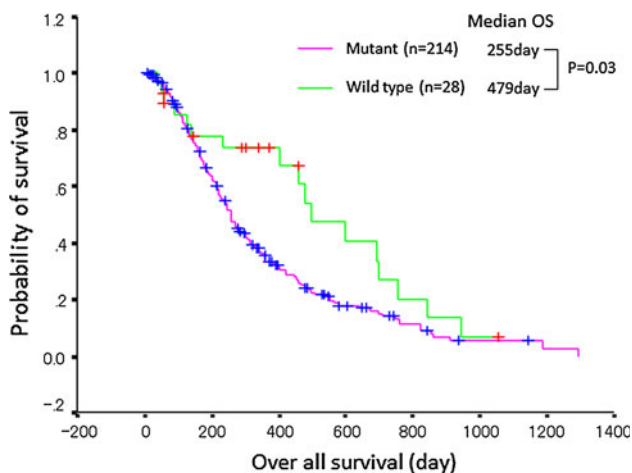
**Table 1** Patient characteristics according to *K-ras* mutation status in unresectable pancreatic cancer

Variables	<i>K-ras</i> gene		<i>P</i> -value	G12D ( <i>n</i> = 92)	G12V ( <i>n</i> = 85)	G12R ( <i>n</i> = 17)	<i>P</i> -value
	Wild-type ( <i>n</i> = 28)	Mutant ( <i>n</i> = 214)					
Gender							
Male	18	128	0.65*	58	51	8	0.46*
Female	10	86		34	34	9	
Age (range)	61 (43–77)	65 (35–84)	0.12**	64 (35–81)	65 (38–84)	66 (48–84)	0.74***
ECOG PS							
3	0	3	0.25*	1	1	1	0.41*
2	0	13		7	3	1	
1	11	54		27	32	3	
0	17	144		57	49	12	
Tumor location							
Head	14	88	0.42*	33	37	7	0.58*
Body, tail	14	126		59	48	10	
CA19-9 (U/ml)	11693.0 (±7320.0)	20787.1 (±6711.7)	0.74**	36302.5 (±14687.1)	8274.0 (±4808.6)	13747.8 (±7465.1)	0.20***
Stage							
Local	12	73	0.34*	31	30	5	0.89*
Metastasis	16	141		61	55	12	
Chemotherapy							
GEM	20	136	0.61*	60	55	10	0.13*
GEM+α	4	37		18	13	3	
Other	3	18		6	5	4	
None	1	23		8	12	0	

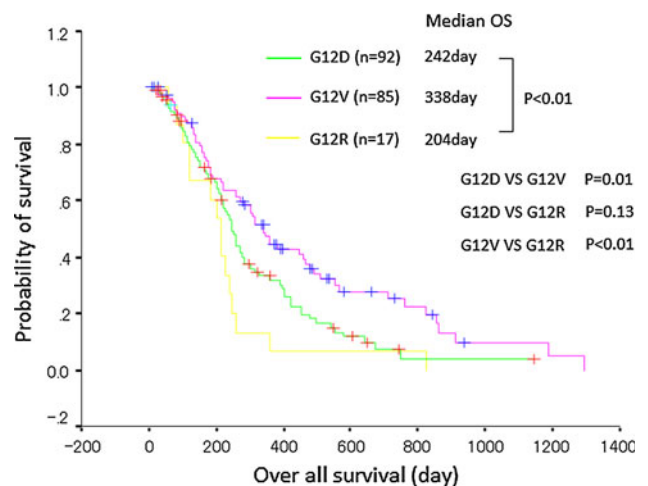
\* $\chi^2$  test

\*\*Mann–Whitney *U* test

\*\*\*Kruskal–Wallis test



**Fig. 1** Overall survival (OS) in all patients was examined with Kaplan–Meier plots and log-rank tests. The median OS was significantly longer in the wild-type group (479 days) than in the mutant group (255 days; *P* = 0.03)



**Fig. 2** Overall survival (OS) in patients who had the *K-ras* mutation subtypes, G12D, G12V, and G12R was examined with Kaplan–Meier plots and log-rank tests. Patients who had the G12V mutation had a significantly longer OS than patients with G12D (*P* = 0.01) or G12R (*P* < 0.01)

**Table 2** Prognostic factors for OS in univariate and multivariate analyses among all patients ( $n = 242$ )

Variables ( $n$ )	Median OS* (day)	Univariate		Multivariate	
		HR** (95 % CI***)	$P$ -value	HR (95 % CI)	$P$ -value
Gender					
Male (146)	302	1.19 (0.88–1.61)	0.27		
Female (96)	256				
Age					
$\geq 65$ (121)	241	0.80 (0.60–1.09)	0.16		
$< 65$ (121)	302				
PS					
$\geq 2$ (16)	103	2.58 (1.46–4.55)	$< 0.01$	1.59 (0.87–2.91)	0.13
$< 2$ (226)	302				
Location					
Head (102)	305	0.81 (0.60–1.10)	0.17		
Body, tail (140)	257				
CA19-9 (U/ml)					
$\geq 1000$ (104)	202	1.95 (1.42–2.69)	$< 0.01$	1.78 (1.28–2.46)	$< 0.01$
$< 1000$ (121)	340				
CRP**** (mg/dl)					
$\geq 3$ (24)	139	1.51 (0.97–2.34)	0.71		
$< 3$ (138)	266				
Stage					
Local (83)	480	2.44 (1.72–3.45)	$< 0.01$	2.26 (1.58–3.24)	$< 0.01$
Metastasis (159)	222				
<i>K-ras</i> gene					
Wild type (28)	479	1.78 (1.08–2.95)	0.03	1.76 (1.03–3.01)	0.04
Mutant (214)	255				

\*OS overall survival, \*\*HR hazard ratio, \*\*\*CI confidence interval, \*\*\*\*CRP C-reactive protein

by subtype, G12D was present in 10 %, G12V in 21 %, and G12R in 6 %. These data suggest that patients with wild-type *K-ras* or G12V have better response rates to chemotherapy. It should be noted, however, that these data were only retrospective, and prospective analysis conforming strictly to Response Evaluation Criteria in Solid Tumors (RECIST) [25] is required.

Compared with previous studies [8–12], the present study included the largest number of patients, and, to the best of our knowledge, it is the first study to analyze OS and prognostic factors according to *K-ras* mutation status and subtypes in only unresectable PC using EUS-FNA materials. However, the present study does have several limitations, such as the retrospective design and patient selection bias.

In univariate analyses, PS, CA19-9, stage, and *K-ras* mutation and subtypes were identified as significant predictors of poor prognosis. In addition, these factors had independent prognostic value in a multivariate analysis without PS. Although various factors other than *K-ras* are also important for prognostication, *K-ras* mutation analysis may be particularly useful for predicting prognosis in PC patients with local-stage, PS 0, or low serum levels of CA19-9. Patients without *K-ras* mutation survived significantly longer than patients with *K-ras* mutation. Among the

patients with *K-ras* mutations, patients with the G12V mutation had a significantly longer OS as compared with the patients with the G12D or G12R mutations. These results are consistent with the results of previous studies [6, 7, 11, 26]. The frequency of *K-ras* mutation in our cohort was very similar to previously reported frequencies, and the distribution of *K-ras* mutation subtypes was also similar to distributions in other studies. The most common alteration in codon 12 of *K-ras* was G12D, followed by G12V and then G12R. Moreover, our results for OS by mutation subtype and the presence or absence of *K-ras* mutation were similar to previously reported results [9–12]. Immervoll et al. [12] previously suggested that mutation subtype could be associated with survival. In their study of resected PC patients, patients with the G12V mutation had a longer median survival period (23.5 months) than patients with G12D (9.5 months), who lived longer than patients with G12R (7.5 months). Likewise, Kawesha et al. [11] also demonstrated a longer median survival period in patients with G12V (12.5 months) than in those with G12D (8.7 months) or G12R (6.7 months). In a study of colorectal cancer patients, Span et al. [27] demonstrated that patients with G12D and G12R tended to have a worse prognosis than patients with G12V.

**Table 3** Prognostic factors for OS in univariate and multivariate analyses according to *K-ras* mutation subtype ( $n=194$ )

Variables ( <i>n</i> )	Median OS* (day)	Univariate		Multivariate	
		HR** (95 % CI***)	<i>P</i> -value	HR (95 % CI)	<i>P</i> -value
<b>Gender</b>					
Male (117)	276	1.39 (0.99–1.95)	0.05		
Female (77)	245				
<b>Age</b>					
≥65 (95)	229	1.63 (0.84–3.20)	0.15		
<65 (99)	222				
<b>PS</b>					
≥2 (15)	103	2.01 (1.05–3.83)	0.03	1.40 (0.72–2.71)	0.32
<2 (179)	268				
<b>Location</b>					
Head (79)	276	1.23 (0.92–1.81)	0.14		
Body, tail (115)	255				
<b>CA19-9 (U/ml)</b>					
≥1000 (86)	202	1.91 (1.34–2.71)	<0.01	1.65 (1.12–2.37)	<0.01
<1000 (108)	322				
<b>CRP**** (mg/dl)</b>					
≥3 (35)	116	1.56 (0.95–2.54)	0.07		
<3 (159)	268				
<b>Stage</b>					
Local (44)	476	2.33 (1.59–3.42)	<0.01	2.12 (1.44–3.14)	<0.01
Metastasis (150)	220				
<b><i>K-ras</i> gene</b>					
G12V (85)	340	1.72 (1.22–2.43)	<0.01	1.60 (1.11–2.28)	0.01
G12D, G12R (109)	241				

\*OS overall survival, \*\*HR hazard ratio, \*\*\*CI confidence interval, \*\*\*\*CRP C-reactive protein

However, in the RASCAL project, which analyzed 2271 colorectal cancer patients from 22 groups in 13 countries, G12V was associated with a greater risk of relapse and death than other mutations [28]. Studies of lung carcinoma have also concluded that patients with the G12V mutation have a poorer prognosis than patients with G12D [29, 30]. These results are supported by sound experimental evidence. The basal GTPase activity of the G12V mutant is one-quarter that of the G12D mutant and one-tenth that of wild-type *K-ras* [31, 32]. In addition, Al-Mulla and Mackenzie [33] found that Rat-1 cells containing G12V mutant *K-ras* were significantly more invasive in vitro than clones containing the G12D mutation or wild-type *K-ras*. The G12V-expressing cells had a greater ability to invade Matrigel than cells expressing the G12D mutant or wild-type *K-ras*. However, although they also pointed out that the biological effects of G12V, G12D, and wild-type *K-ras* cannot be assumed to be the same, mutant *K-ras* and wild-type *K-ras* have been associated with different degrees of biological aggression in the abovementioned basic experiments, which may also be reflected in prognosis in PC patients.

In conclusion, we analyzed prognostic factors including *K-ras* mutation status and subtypes in PC patients. This

study is the largest of its kind yet to be published; also, this is the first analysis of unresectable PC performed with tissue obtained by EUS-FNA. Differences in the survival of PC patients cannot be completely explained on the basis of *K-ras* mutation status and subtype. However, according to our study, the *K-ras* mutation status and specific subtypes may be important factors associated with survival in PC patients. Our results need to be validated by future prospective studies with the analysis of more prognostic factors and more detailed oncogene analyses.

**Conflict of interest** The authors declare that they have no conflicts of interest.

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